

# Isolation of Butanol- and Isobutanol-Tolerant Bacteria and Physiological Characterization of Their Butanol Tolerance

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Despite their importance as a biofuel production platform, only a very limited number of butanol-tolerant bacteria have been identified thus far. Here, we extensively explored butanol- and isobutanol-tolerant bacteria from various environmental samples. A total of 16 aerobic and anaerobic bacteria that could tolerate greater than 2.0% (vol/vol) butanol and isobutanol were isolated. A 16S rRNA gene sequencing analysis revealed that the isolates were phylogenetically distributed over at least nine genera: *Bacillus*, *Lysinibacillus*, *Rummeliibacillus*, *Brevibacillus*, *Coprothermobacter*, *Caloribacterium*, *Enterococcus*, *Hydrogenoanaerobacterium*, and *Cellulosimicrobium*, within the phyla *Firmicutes* and *Actinobacteria*. Ten of the isolates were phylogenetically distinct from previously identified butanol-tolerant bacteria. Two relatively highly butanol-tolerant strains CM4A (aerobe) and GK12 (obligate anaerobe) were characterized further. Both strains changed their membrane fatty acid composition in response to butanol exposure, i.e., CM4A and GK12 exhibited increased saturated and cyclopropane fatty acids (CFAs) and long-chain fatty acids, respectively, which may serve to maintain membrane fluidity. The gene (*cfa*) encoding CFA synthase was cloned from strain CM4A and expressed in *Escherichia coli*. The recombinant *E. coli* showed relatively higher butanol and isobutanol tolerance than *E. coli* without the *cfa* gene, suggesting that *cfa* can confer solvent tolerance. The exposure of strain GK12 to butanol by consecutive passages even enhanced the growth rate, indicating that yet-unknown mechanisms may also contribute to solvent tolerance. Taken together, the results demonstrate that a wide variety of butanol- and isobutanol-tolerant bacteria that can grow in 2.0% butanol exist in the environment and have various strategies to maintain structural integrity against detrimental solvents.

Due to fluctuations in the price of crude oil and an increase in environmental concerns, microbial fuel production from renewable feedstock has become a global priority. *n*-Butanol (hereafter referred to as butanol) is considered to be a good alternative to the traditional biofuel ethanol due to the advantages of butanol's high energy content, high octane rating, low volatility, and miscibility with gasoline and diesel oil (1, 2).

Butanol is produced biologically through an acetone-butanol-ethanol (ABE) fermentation process by obligate anaerobic clostridia (3). Recently, vigorous efforts have been made to develop alternative processes to generate C<sub>3</sub>- to C<sub>5</sub>-chain alcohols, including butanol and 2-methylpropan-1-ol (hereafter referred to as isobutanol), through metabolic engineering using *Escherichia coli* as the platform microorganism (4). However, production is very limited due to the high toxicity of butanol and isobutanol to microorganisms (5, 6). These solvents are known to cause an increase in plasma membrane fluidity by intercalating into the membrane and breaking the hydrogen bonds between lipid tails, resulting in a loss of membrane potential and a decline in cell growth (7, 8, 9). In fact, the known butanol-producing microorganisms, including clostridia, genetically engineered *E. coli*, and *Saccharomyces cerevisiae*, are highly sensitive to butanol (10, 11, 12), and this toxicity has long been a critical issue in practical biobutanol production.

To date, only a few butanol-tolerant bacterial species with the ability to grow in greater than 2.0% (vol/vol) butanol have been found (5, 10, 13, 14, 15). The previously identified butanol-tolerant microorganisms were predominantly screened from culture collections that included well-known strains that tolerate other

organic solvents (e.g., toluene and benzene) (5, 10, 14). This strategy was effective but resulted in the collection of taxonomically limited butanol-tolerant bacteria, such as the genera *Clostridium*, *Pseudomonas*, *Zymomonas*, *Bacillus*, *Lactobacillus*, and *Enterococcus* (5, 7, 10, 13, 14, 15). Furthermore, as there have been very few attempts to isolate butanol-tolerant bacteria from natural environments, very little is known about the butanol-tolerant bacteria that are present in nature.

In this study, we sought to extensively screen butanol- and isobutanol-tolerant microorganisms that can grow in greater than 2.0% butanol from various environmental samples and investigated the phylogenetic positions and tolerance to butanol and isobutanol of the microorganisms. Among those isolates, two strains that showed a relatively high tolerance were further characterized by examining cell surface structures, fatty acid compositions, and genes associated with butanol tolerance.

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## MATERIALS AND METHODS

**Sampling, enrichment, and isolation.** Butanol- and isobutanol-tolerant microorganisms were enriched in the presence of these solvents. Cultivation was performed under both aerobic and strictly anaerobic conditions. To prevent evaporation of the solvents, a 50-ml, tightly capped conical tube (for aerobic cultivation) and 50-ml serum vial sealed with a butyl rubber stopper and aluminum crimp (for strictly anaerobic cultivation) were used. Evaporation was negligible during the cultivation period under both the aerobic and anaerobic conditions and thus did not affect the evaluation of solvent tolerance.

To enrich and isolate aerobic bacteria, samples were collected from freshwater sediments, grease-contaminated soils, cabbage-field soils, vegetable wastes, and composts in Ibaraki Prefecture, Japan. After the samples were sonicated in water and centrifuged at  $400 \times g$  for 5 min, the supernatant was inoculated into fresh medium containing butanol (0.1 to 3.0% [vol/vol]); the butanol-containing medium consisted of (per liter) 5 g glucose, 1 g tryptone, 0.5 g yeast extract, 1 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.75 g  $\text{KH}_2\text{PO}_4$ , and 0.78 g  $\text{K}_2\text{HPO}_4$  supplemented with 7 ml basal salt solution and 1 ml vitamin solution, as described previously (16). Two different aerobic enrichments, short- and long-term cultivations, were performed. For short-term cultivation, 50  $\mu\text{l}$  of inoculum was added to 2 ml of fresh medium containing 0.5 to 3.0% butanol. After incubation at 30°C with shaking for 48 h, the cultures were spread onto agar plates with the same medium containing 2.0% butanol, and colonies were isolated and purified. For long-term cultivation (3 to 9 months in total), 1 ml of each inoculum was added to 50 ml of fresh medium containing 0.1% butanol. After incubation at room temperature with shaking for 48 to 72 h, 2 to 3% of the culture fluid was transferred into fresh medium containing 1.0% butanol and incubated for 5 days. The culture was then repeatedly transferred into fresh medium with increasing concentrations of butanol of up to 9.0% in a stepwise manner. After consecutive transfers, the cultures were spread onto agar plates as described above.

To enrich and isolate strictly anaerobic bacteria, samples for enrichment were collected from oil-contaminated soils, thermophilic and mesophilic anaerobic digesters, bovine rumens, hot springs, and bovine manure composts in Hokkaido Prefecture, Japan. The oil-contaminated soil or bovine manure compost samples were mixed well with 10 mM phosphate-buffered 150 mM NaCl. A portion of a 5-ml sample or slurry was inoculated into fresh medium containing 2.0% or 5.0% butanol or isobutanol. The medium was prepared based on a modified Widdel medium (17) with the following composition (per liter), as described previously (18): 5 g glucose, 1 g yeast extract, 0.53 g  $\text{NH}_4\text{Cl}$ , 0.14 g  $\text{KH}_2\text{PO}_4$ , 0.2 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.15 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 2.52 g  $\text{NaHCO}_3$  supplemented with 1 ml selenium and tungsten solution, 1 ml trace element solution, and 2 ml vitamin solution. Cultivation was performed in 50-ml serum vials containing 20 ml of medium under an atmosphere of  $\text{N}_2/\text{CO}_2$  (80:20) at 37°C or 55°C. The cultures were transferred to fresh media five times at intervals of approximately 1 month. The Hungate roll tube technique (19) was used to isolate single colonies using the above-mentioned medium solidified with 2% Noble agar. Each colony was inoculated into a liquid medium containing 2.0% butanol to verify tolerance to butanol.

**Phylogenetic analysis based on 16S rRNA gene sequences.** The 16S rRNA gene sequences (*E. coli* positions 28 to 1491) of each isolate were determined as previously described (20). All sequences were aligned with their relatives using ARB software (21) and the SILVA database (22). A phylogenetic tree was constructed by the neighbor-joining method (23) using the MEGA 4.0 program (24). The robustness of the tree's topology was assessed by a bootstrap analysis (25) based on 1,000 replications.

**Butanol and isobutanol tolerance assay.** To obtain better growth for determining solvent tolerance and physiological traits, the media for the aerobic and anaerobic solvent-tolerant isolates were altered as follows. For the aerobic strains, 5 g/liter glucose, 20 g/liter tryptone, 5 g/liter yeast extract, 1.5 g/liter  $\text{KH}_2\text{PO}_4$ , and 1.56 g/liter  $\text{K}_2\text{HPO}_4$  were supplemented with 7 ml basal salt solution and 1 ml vitamin solution (16). For the anaerobic strains, 10 g/liter glucose, 5 g/liter yeast extract, 0.53 g/liter

$\text{NH}_4\text{Cl}$ , 0.14 g/liter  $\text{KH}_2\text{PO}_4$ , 0.2 g/liter  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.15 g/liter  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 2.52 g/liter  $\text{NaHCO}_3$  were supplemented with 1 ml selenium and tungsten solution, 1 ml trace element solution, and 2 ml vitamin solution (18). The cultivation temperature for each isolate was the same temperature used in the enrichment.

Tolerance to butanol and isobutanol of the isolates was assessed based on cellular growth in the presence of the solvents as previously described (5, 10, 13, 15). In particular, butanol-tolerant bacteria were defined in this study as those that could grow in greater than 2.0% butanol, as all of the previously identified butanol-tolerant microorganisms can grow in at least 2.0% butanol (5, 10, 13, 14, 15). The maximum concentrations of butanol and isobutanol that allowed the isolates to grow were determined. Cells grown without solvent were inoculated into fresh liquid media amended with butanol or isobutanol at a concentration of 1.0 to 5.5% in increments of 0.5%. The optical density at 600 nm ( $\text{OD}_{600}$ ) was monitored using a spectrophotometer, and the specific growth rates were calculated from the linear range of exponential growth. The concentration of butanol or isobutanol in culture was measured by high-performance liquid chromatography equipped with a cation-exchange column and a refractive index (RI) detector. To examine cellular adaptation to butanol, all the isolates were grown with 2.0% butanol and inoculated into the subsequent medium. After 2 to 15 successive transfers, the butanol tolerance of the isolates was evaluated by the same methods as described above. Ultimately, two isolates (strains CM4A and GK12) with a relatively high butanol tolerance were further characterized as described below.

**TEM.** Changes in the cell surface morphology of strains CM4A and GK12 in the late exponential growth phase and grown with or without butanol were observed by transmission electron microscopy (TEM) (Hitachi H-7600) (26). The thickness of the extracellular capsule of strain CM4A was measured from the transmission electron micrographs of 10 randomly selected cells (two spots per single cell;  $n = 20$ ).

**Cell surface hydrophobicity measurement.** The cell surface hydrophobicity of strain CM4A grown with or without butanol was assessed by the bacterial adhesion to hydrocarbon (BATH) test (27). In brief, 0.2 ml of organic solvent was added to 5 ml of a late-exponential-phase cell suspension, which was adjusted to an  $\text{OD}_{600}$  of 1.0. The solution was then vortex agitated for 30 s and left to stand for 15 min to allow separation. Butanol, *n*-hexane, *n*-tetradecane, toluene, and xylene were used as the solvents in the assay. The percentage of cells that adhered to the solvent was calculated by the following formula:  $[1 - (\text{OD}_{600}$  of aqueous phase after mixing/ $\text{OD}_{600}$  of initial suspension)]  $\times 100$  (28).

**Fatty acid analysis.** An analysis of whole-cell lipid extracts from strains CM4A and GK12 grown with or without butanol was performed using previously described methods (29). Briefly, cells in the late exponential phase of growth were directly methanolized. The products containing fatty acid methyl esters were then extracted with *n*-hexane and analyzed by gas chromatography-mass spectrometry (M7200A GC/3DQMS system; Hitachi, Japan).

**Cloning and expression of the cyclopropane fatty acid (CFA) synthase gene (*cf*a) in *E. coli*.** The *cf*a gene of strain CM4A was amplified and cloned into the pET-28b expression vector (Novagen) to produce an N-terminal His<sub>6</sub>-tagged fusion protein. The primers used for the PCR amplification, direct sequencing, and cloning of the *cf*a gene are listed in Table S1 in the supplemental material. *E. coli* DH5 $\alpha$  cells were transformed and subsequently plated onto LB medium supplemented with 30  $\mu\text{g}/\text{ml}$  kanamycin. The expression and activity of the recombinant protein in the positive clone, designated *E. coli*/pCFA, were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by the above-described fatty acid analysis, respectively. *E. coli*/pCFA and the negative-control clone *E. coli*/pET28, which was transformed with an empty pET-28b vector, were subjected to the solvent tolerance assay as described above. Briefly, the cells were grown on LB medium containing 0.7 to 1.0% butanol or isobutanol, 0.01 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG), and 30  $\mu\text{g}/\text{ml}$  kanamycin.

TABLE 1 Characteristics of the isolates

Bacterial type and strain	Origin	Enrichment conditions <sup>a</sup>	Phylum	Closest relative species (accession no.)	Similarity (%)	Tolerance (%) <sup>b</sup>	
						Butanol	Isobutanol
Butanol-tolerant bacteria isolated under aerobic conditions							
CM4A	Grease-contaminated soil	30°C/4 mo/4.0%	Firmicutes	<i>Enterococcus faecalis</i> (AB012212)	99.6	3.5	4.0
CM9A	Grease-contaminated soil	30°C/9 mo/9.0%	Firmicutes	<i>Rummeliibacillus pycnus</i> (AB271739)	98.4	2.0	2.5
SK4A	Freshwater sediment	30°C/3 mo/4.0%	Firmicutes	<i>Bacillus amyloliquefaciens</i> (AB255669)	99.6	2.0	2.5
SK4D	Freshwater sediment	30°C/4 mo/4.0%	Firmicutes	<i>Enterococcus faecalis</i> (AB012212)	99.6	2.5	3.0
SK5A	Freshwater sediment	30°C/7 mo/5.0%	Firmicutes	<i>Enterococcus faecalis</i> (AB012212)	99.5	3.0	3.5
SK7A	Freshwater sediment	30°C/9 mo/7.0%	Firmicutes	<i>Lysinibacillus xylanilyticus</i> (FJ477040)	98.5	2.5	3.0
SK9A	Freshwater sediment	30°C/9 mo/9.0%	Firmicutes	<i>Brevibacillus reuszeri</i> (AB112715)	99.8	2.0	2.5
FW5A	Vegetable waste	30°C/2 days/0.5%	Firmicutes	<i>Bacillus amyloliquefaciens</i> (AB255669)	97.9	2.0	2.5
CP3A	Cabbage-field soil	30°C/2 days/3.0%	Firmicutes	<i>Bacillus mycoides</i> (AB021192)	98.3	2.5	3.0
CY2C	Compost	30°C/2 days/2.0%	Actinobacteria	<i>Cellulosimicrobium cellulans</i> (X83809)	98.1	2.0	2.5
Butanol-tolerant bacteria isolated under anaerobic conditions							
GK3	Thermophilic anaerobic digester	37°C/5 mo/2.0%	Firmicutes	<i>Garciella nitratreducens</i> (AY176772)	92.8	2.0	2.5
GK5	Thermophilic anaerobic digester	55°C/4 mo/2.0%	Firmicutes	<i>Coprothermobacter proteolyticus</i> (X69335)	98.8	2.0	2.0
GK12	Thermophilic anaerobic digester	37°C/5 mo/2.0%	Firmicutes	<i>Eubacterium cylindroides</i> (L34617)	91.2	3.0	3.5
YN1	Oil-contaminated soil	37°C/5 mo/2.0%	Firmicutes	<i>Caloribacterium cisternae</i> (JF262044)	96.7	2.0	2.0
YN3	Oil-contaminated soil	37°C/5 mo/2.0%	Firmicutes	<i>Hydrogenoanaerobacterium saccharovorans</i> (EU158190)	98.0	2.0	2.0
YN5	Oil-contaminated soil	37°C/4 mo/2.0%	Firmicutes	<i>Clostridium pasteurianum</i> (FR870440)	93.6	2.5	3.0

<sup>a</sup> The conditions of the enrichment cultures are given as follows: temperature/culture period/final butanol concentration.

<sup>b</sup> The values represent the maximum butanol or isobutanol concentration allowing growth and were based on three independent replicates. The temperature at which tolerance was tested was the same temperature used for enrichment.

**Nucleotide sequence accession numbers.** The GenBank/EMBL/DBJ accession numbers of the 16S rRNA gene sequences of the isolates are AB669587 to AB669596 and AB537978 to AB537983, and the accession number of the *cf*a gene of strain CM4A is AB669597.

## RESULTS AND DISCUSSION

**Isolation of butanol- and isobutanol-tolerant bacteria.** After enrichment in the presence of butanol under aerobic and anaerobic conditions, a total of 16 microorganisms that could grow in the presence of greater than 2.0% butanol and isobutanol were isolated from various environmental samples (Table 1). All isolates comprised heterotrophic mesophilic bacteria, except for a thermophilic bacterium, strain GK5. To date, no thermophilic microorganisms tolerating butanol have been found. The obligate anaerobic isolates (GK3, GK5, GK12, YN1, YN3, and YN5) were obtained from either thermophilic anaerobic digesters or oil-contaminated soil. In contrast, aerobic strains (CM4A, CM9A, SK4A, SK4D, SK5A, SK7A, SK9A, FW5A, CP3A, and CY2C) were isolated from all the samples used in this study, even freshwater sediments and cabbage-field soils in which organic solvents were likely not present.

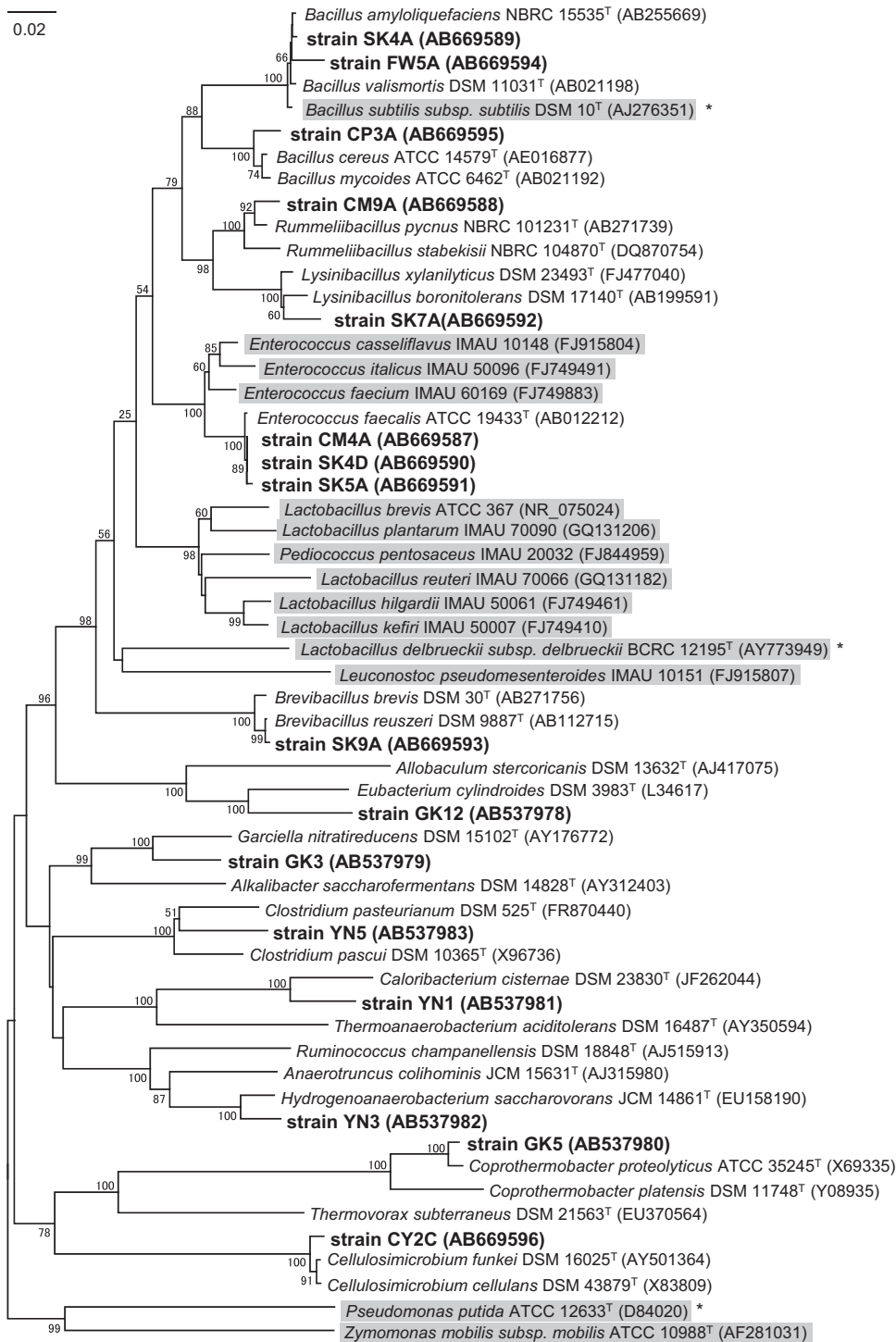
**Phylogenetic identification of the butanol-tolerant microorganisms obtained.** The phylogenetic analysis based on 16S rRNA gene sequencing indicated that the isolates obtained were affiliated with the genera *Bacillus*, *Lysinibacillus*, *Brevibacillus*, *Rummeliibacillus*, *Coprothermobacter*, *Caloribacterium*, *Enterococcus*, and *Hydrogenoanaerobacterium* within the phylum Firmicutes and the genus *Cellulosimicrobium* within the phylum Actinobacteria (Table 1). Strains CM4A, SK4D, and SK5A, belonging to the genus *Enterococcus*, exhibited a relatively higher tolerance than did the other isolates. This finding is consistent with the results of a previous study showing that many lactic acid bacteria, including *Enterococcus* species, can tolerate butanol (5, 15). Similarly, strains SK4A, FW5A, and CP3A were closely related to the known buta-

nol-tolerant bacterium *Bacillus subtilis* (13). In contrast, the other 10 isolates were phylogenetically distinct from the previously identified butanol-tolerant bacteria (Fig. 1). In particular, strains GK3, GK12, and YN5 were phylogenetically novel, showing less than 94% sequence similarity to the most closely related species. This result indicates that there is a wider variety of butanol- and isobutanol-tolerant bacteria that can grow in the presence of greater than 2.0% solvent than previously recognized.

**Solvent tolerance of the isolates.** To further evaluate the ability of the isolates to tolerate butanol and isobutanol, the maximum concentrations of solvents that allowed the isolates to grow were determined. Most of the isolates (all of the aerobic strains and three anaerobic strains [GK3, GK12, and YN5]) grew slightly better with isobutanol than with butanol (Table 1). None of the isolates tested assimilated or degraded butanol and isobutanol (data not shown).

Of the isolates, the aerobic strain CM4A exhibited the highest tolerance, growing in the presence of 3.5% butanol and 4.0% isobutanol. The anaerobic isolate, strain GK12, also showed a relatively higher tolerance, with the ability to grow in 3.0% butanol and 3.5% isobutanol. The tolerance levels of the two strains were comparable or even greater than the tolerance of previously identified butanol-tolerant microorganisms (5). More importantly, both strains showed clear exponential growth and reached high cell densities, even in the presence of 2.5% butanol (Fig. 2), which was not observed for the previously reported butanol-tolerant bacteria (5, 15). Therefore, we selected strains CM4A and GK12 for further analyses.

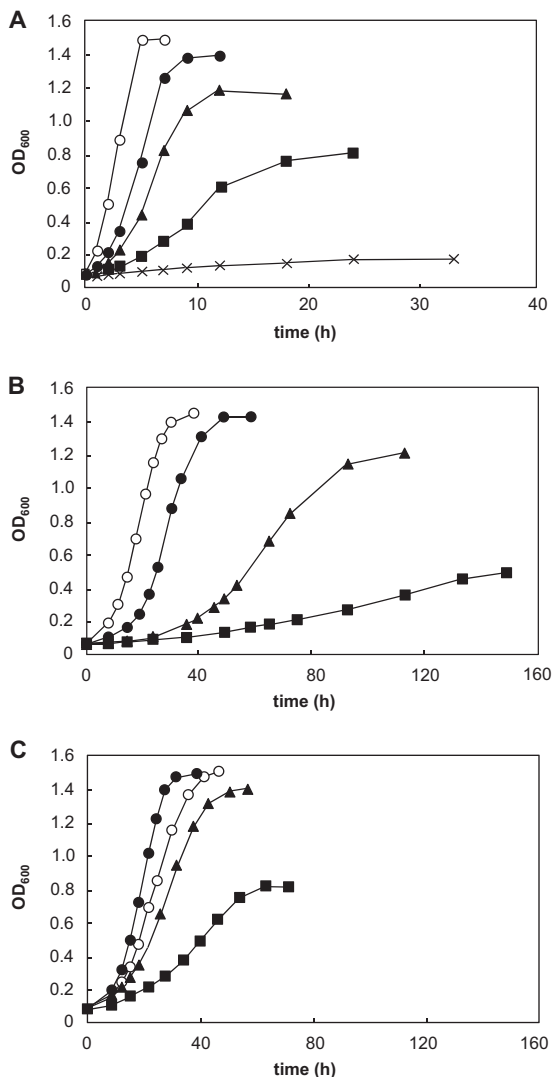
**Morphological characterization of strains CM4A and GK12.** The cell morphologies were investigated by TEM. As shown in Fig. 3, changes in the cell surface properties in the presence of a high butanol concentration (2.0%) were evident in strain CM4A. The cells exhibited a diplococcus shape with a capsule structure



**FIG 1** Neighbor-joining tree based on 16S rRNA gene sequences, showing the relationship between the isolates (boldface type), their relatives, and other butanol-tolerant bacteria (shaded clusters). Due to the lack of sequences for butanol-tolerant *Bacillus subtilis* (13), *Lactobacillus delbrueckii* (10), and *Pseudomonas putida* (14) strains, as indicated by asterisks, their type strains are shown in the tree. The bootstrap values that were above 50% are shown at the nodes. Bar, 0.02 substitution per nucleotide position.

(Fig. 3A and C), and the thickness of the extracellular capsule increased 2-fold when the cells were grown with butanol ( $P < 0.01$  by  $t$  test) (Fig. 3B and D). To date, these changes in cell surface properties in response to butanol exposure have not been re-

ported. The BATH test (27) was performed to clarify whether the cell surface hydrophobicity of strain CM4A was altered by the increased extracellular capsule thickness. Cells grown with 2% butanol exhibited a significantly lower affinity for butanol and

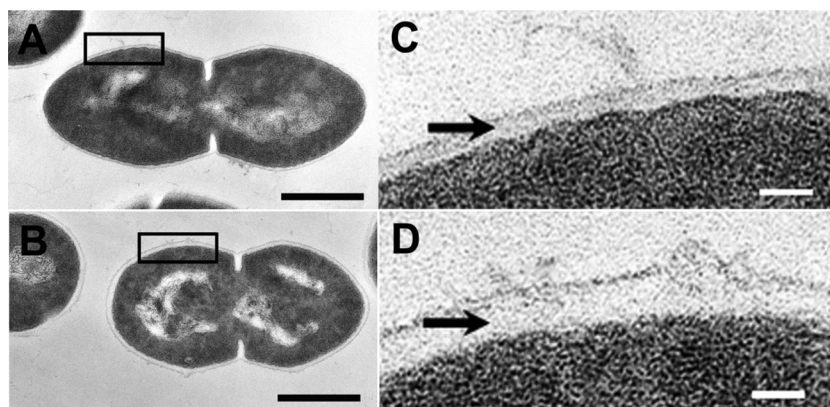


**FIG 2** Effect of butanol on the growth of strain CM4A (A), strain GK12 (B), and the butanol-adapted cells of strain GK12 (C). Butanol concentrations of 0% (○), 2% (●), 2.5% (▲), 3.0% (■), and 3.5% (×) were tested. The values represent the means of triplicate experiments.

other organic solvents than did the cells grown without butanol ( $P < 0.01$  by  $t$  test) (see Table S2 in the supplemental material). This result suggests that the changes in the capsule structure of strain CM4A decrease the cell surface hydrophobicity and thus may function as a physical defense against butanol. Previous studies have reported that the solvent-tolerant microorganisms *Rhodococcus rhodochrous* and *Staphylococcus* sp. produce extracellular hydrophilic compounds that may prevent hydrophilic solvents, such as *n*-hexadecane and toluene, from intercalating into the membrane (30, 31). In contrast to strain CM4A, such a change in morphology was not observed in strain GK12 (data not shown).

**Changes in the fatty acid compositions of strains CM4A and GK12 in the presence of butanol.** To elucidate the underlying mechanism of solvent tolerance, we further investigated the physiological response to butanol of strains CM4A and GK12. Exposure to solvents impairs the integrity and stability of the cytoplasmic membrane, and several solvent-tolerant microorganisms are known to reestablish membrane fluidity and decrease solvent permeability by altering their membrane lipid compositions (7, 32, 33). Thus, to evaluate the responses of the cell membrane to high concentrations of butanol, changes in the fatty acid compositions of the highly solvent-tolerant strains CM4A and GK12 were examined at a concentration of up to 2.5% butanol.

In the absence of butanol, strain CM4A contained saturated fatty acids (44% of total), together with unsaturated fatty acids (56%), such as palmitic acid ( $C_{16:0}$ ) and *cis*-vaccenic acid ( $C_{18:1\omega7c}$ ). When the cells were grown with butanol, the proportion of  $C_{16:0}$  and CFAs (*cis*-11,12-methylene octadecanoic acid [cyclo- $C_{19:0}$ ]) increased, whereas the proportion of  $C_{18:1\omega7c}$  decreased, resulting in an increase in the proportion of total saturated fatty acids and CFAs by up to 56% (Table 2). Although the *cis*-hexadecenoic acid ( $C_{16:1\omega7c}$ ) level relatively increased with 2.0% butanol but decreased with 2.5%, the total unsaturated fatty acid proportion tended to decrease in a butanol dose-dependent manner (Table 2). In contrast, strain GK12 did not contain unsaturated fatty acids but rather saturated fatty acids and saturated 1,1-dimethoxy alkanes (DMAs) (34). In the presence of butanol, the proportions of longer-chain fatty acids (stearic acid [ $C_{18:0}$ ] and arachidic acid [ $C_{20:0}$ ]) significantly increased (Table 3). Although the proportion of  $C_{18:0}$  DMAs relatively increased with 2.0% butanol but de-



**FIG 3** (A to D) Transmission electron micrographs of strain CM4A in the absence (A and C) and presence (B and D) of 2.0% butanol. The transmission electron micrographs in panels C and D are magnified views of the black boxes in panels A and B, respectively. The black arrows point to the positions of the capsule. The average capsule thicknesses were  $17 \pm 3.7$  nm and  $32 \pm 3.7$  nm in the absence and presence, respectively, of 2.0% butanol ( $P < 0.01$  by  $t$  test). Bars, 0.5 μm (A and B) and 50 nm (C and D).

**TABLE 2** Changes in the fatty acid composition of strain CM4A in the presence of 2.0% and 2.5% butanol

Fatty acid <sup>a</sup>	Fatty acid composition <sup>b</sup> (%) of strain CM4A grown with:		
	No solvents	2.0% butanol	2.5% butanol
C <sub>9:1</sub> <sup>c</sup>	ND	ND	1.1 ± 0.3
C <sub>14:0</sub>	5.7 ± 0.2	6.9 ± 0.6	6.6 ± 0.2
C <sub>14:1ω7c</sub>	0.6 ± 0.1	0.8 ± 0.1	ND
C <sub>15:0</sub>	0.1 ± 0.0	0.1 ± 0.0	ND
C <sub>16:0</sub>	35.7 ± 1.3	41.0 ± 0.8	45.8 ± 0.7
C <sub>16:1</sub> <sup>c</sup>	ND	ND	0.9 ± 0.0
C <sub>16:1ω7c</sub>	8.9 ± 0.2	9.7 ± 0.4	7.9 ± 0.3
C <sub>18:0</sub>	1.9 ± 0.1	1.2 ± 0.0	1.7 ± 0.2
C <sub>18:1ω7c</sub>	46.4 ± 1.5	38.5 ± 1.6	33.6 ± 0.4
Cyclo-C <sub>19:0</sub>	0.6 ± 0.1	1.8 ± 0.2	2.4 ± 0.1
Total saturated fatty acids <sup>d</sup>	43.4 ± 1.4	49.2 ± 1.3	54.1 ± 0.5

<sup>a</sup> Abbreviations: C<sub>X:YωZc</sub>, a fatty acid containing X carbon atoms with Y double bonds at position Z, counted from the methyl terminus in the *cis* configuration; cyclo-C<sub>19:0</sub>, *cis*-11,12-methylene octadecanoic acid.

<sup>b</sup> Each fatty acid composition value is given as a percentage of the whole-cell lipids. Values are means ± standard deviations of three independent measurements. ND, not detected.

<sup>c</sup> The double-bond positions of C<sub>9:1</sub> and C<sub>16:1</sub> were not identified.

<sup>d</sup> Myristic acid (C<sub>14:0</sub>), pentadecanoic acid (C<sub>15:0</sub>), palmitic acid (C<sub>16:0</sub>), and stearic acid (C<sub>18:0</sub>) were added together to calculate total saturated fatty acids.

creased with 2.5%, the total proportion of alkyl groups of a length of 18 increased in a butanol dose-dependent manner (Table 3). Because increases in the acyl chain length and proportion of saturated fatty acids and the cyclization of unsaturated fatty acids promote more-rigid membrane structures (35, 36), these changes in response to butanol exposure in both strains may compensate for the increased membrane fluidity imposed by butanol. Other microorganisms, such as *Clostridium acetobutylicum* and *Pseudomonas putida*, have also been reported to maintain membrane rigidity by increasing both the mean length of the acyl chain and the proportion of saturated fatty acids and by synthesizing CFAs

in the presence of solvents (37, 38, 39, 40), which is in accordance with our findings.

Overall, butanol dose-dependent changes in the saturated and CFA levels and acyl chain length in strains CM4A and GK12, respectively, were clearly observed (Tables 2 and 3). In particular, strain GK12 exhibited a more marked alteration in membrane composition than strain CM4A. In general, an increase in the length of the acyl chain has a smaller effect on the fluidity of the lipid bilayer than does the saturation of fatty acids (36). Because strain GK12 lacked unsaturated fatty acids, this strain would have to markedly alter the length of the acyl chain of its saturated fatty acids to maintain membrane fluidity.

**Heterologous expression of the *cfa* gene derived from strain CM4A improved solvent tolerance in *E. coli*.** The results of fatty acid analysis indicated that CFAs may contribute to the butanol tolerance of strain CM4A. To verify this phenomenon, we examined whether the cyclization of unsaturated fatty acids among the membrane lipids could enhance the tolerance of *E. coli* to butanol and isobutanol. Recombinant strain *E. coli*/pCFA was generated by the introduction of the *cfa* gene derived from strain CM4A. The deduced 388-amino-acid sequence resulting from the *cfa* gene (AB669597) of strain CM4A is 36% identical to the *E. coli* *cfa* gene (AM946981) product. The expression of the recombinant protein and the increase in the proportion of CFAs (cyclo-C<sub>19:0</sub>) were confirmed by SDS-PAGE and fatty acid analysis, respectively (see Fig. S2 and Table S3 in the supplemental material). A solvent tolerance assay showed that the relative growth rate of recombinant *E. coli*/pCFA in the presence of butanol or isobutanol was significantly higher than the growth rate of the negative-control strain *E. coli*/pET28 ( $P < 0.01$  by *t* test) (Fig. 4; see Fig. S1 in the supplemental material). These findings strongly demonstrate that CFA synthesis due to *cfa* gene transformation enhanced the tolerance of *E. coli* to butanol and isobutanol. CFA synthase is known to directly modify unsaturated fatty acids in the membrane and therefore does not require energy or carbon for *de novo* fatty acid synthesis (35). The enhancement of butanol tolerance by reinforcing CFA synthesis thus did not compete with butanol production, at least in terms of energy and carbon consumption. Introduction

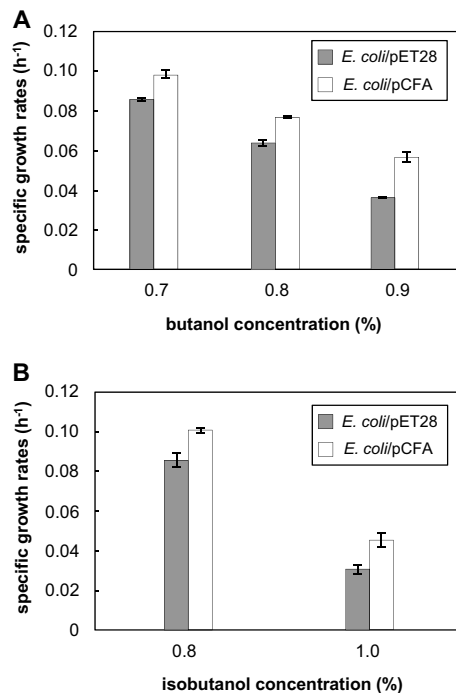
**TABLE 3** Changes in the fatty acid and DMA compositions of nonadapted and butanol-adapted cells of strain GK12 in the presence of butanol

Fatty acid or DMA <sup>a</sup>	Fatty acid or DMA composition <sup>b</sup> (%) of strain GK12						
	Nonadapted cells grown with:			Butanol-adapted cells <sup>c</sup> grown with:			
	No solvents	2.0% butanol	2.5% butanol	No solvents	2.0% butanol	2.5% butanol	3.0% butanol
C <sub>14:0</sub>	24.5 ± 1.1	6.6 ± 0.5	1.5 ± 0.8	27.0 ± 1.6	26.1 ± 0.4	5.2 ± 0.7	1.1 ± 0.4
C <sub>16:0</sub>	23.5 ± 1.4	23.7 ± 2.2	21.6 ± 1.1	25.1 ± 0.3	27.8 ± 0.6	31.2 ± 0.9	19.2 ± 0.4
C <sub>18:0</sub>	10.8 ± 0.6	30.4 ± 0.7	42.1 ± 1.3	3.8 ± 0.5	5.2 ± 0.5	35.2 ± 0.2	48.1 ± 0.8
C <sub>20:0</sub>	2.8 ± 0.6	10.1 ± 1.3	14.1 ± 1.3	2.6 ± 0.5	4.7 ± 0.5	6.6 ± 0.2	19.4 ± 0.6
C <sub>14:0</sub> DMA	4.9 ± 1.3	0.6 ± 0.2	ND	5.5 ± 0.5	2.7 ± 1.2	ND	ND
C <sub>16:0</sub> DMA	18.0 ± 1.7	1.8 ± 0.5	1.6 ± 0.4	10.3 ± 1.3	4.2 ± 0.3	2.1 ± 0.3	1.1 ± 0.4
C <sub>18:0</sub> DMA	15.5 ± 0.8	26.8 ± 2.2	19.1 ± 1.9	25.7 ± 0.6	29.3 ± 0.5	19.6 ± 0.4	11.2 ± 0.9
Total C <sub>14</sub>	29.4 ± 1.4	7.2 ± 0.4	1.5 ± 0.7	32.5 ± 1.4	28.8 ± 1.1	5.2 ± 0.7	1.1 ± 0.4
Total C <sub>16</sub>	41.5 ± 1.9	25.5 ± 2.0	23.3 ± 1.0	35.5 ± 1.2	32.0 ± 0.6	33.3 ± 0.8	20.3 ± 0.5
Total C <sub>18</sub>	26.3 ± 0.9	57.3 ± 2.0	61.2 ± 2.0	29.4 ± 0.7	34.5 ± 0.6	54.8 ± 0.4	59.3 ± 1.0

<sup>a</sup> Abbreviations: C<sub>X:Y</sub>, fatty acid containing X carbon atoms with Y double bonds; total X, the sum of fatty acids and DMAs with an acyl chain length of X.

<sup>b</sup> Each fatty acid composition value is shown as a percentage of whole-cell lipids. Values are means ± standard deviations of three independent measurements. ND, not detected.

<sup>c</sup> Cell adaptation was achieved by 15 consecutive passages with 2.0% butanol.



**FIG 4** Butanol (A) and isobutanol (B) tolerance of *E. coli/pCFA* and the control strain *E. coli/pET28* showing the differences in growth rates in the presence of butanol and isobutanol. To induce heterologous gene expression, 0.01 mM IPTG was added to each strain. Values are means  $\pm$  standard deviations (SD) (error bars) of triplicate experiments. The growth rates of the *E. coli/pCFA* and *E. coli/pET28* strains without solvent were equal.

of the *cfb* gene into a candidate microbial platform may serve as a new strategy to improve butanol production efficiency.

**Mechanisms involved in the butanol adaptation of strain GK12.** Microorganisms are known to adapt to organic solvents during exposure to nonlethal levels of organic solvents (14, 41). To verify whether the isolates could adapt to butanol, the microorganisms were subjected to successive subculturing with nonlethal concentrations of butanol. Only strain GK12 exhibited significant adaptation after 15 consecutive passages in the presence of 2.0% butanol (see Fig. S3 in the supplemental material). Interestingly, the butanol-treated GK12 cells grew even faster in the presence of 2.0% butanol than in the absence of butanol (Fig. 2C and Fig. S3 in the supplemental material). In addition, when the butanol-treated cells were grown in 2.5% or 3.0% butanol, the cell population density in the stationary phase was higher than that of the untreated cells, clearly demonstrating the ability of the cells to adapt to butanol. Conversely, the enhanced tolerance was completely abolished (data not shown) by repeatedly culturing the butanol-treated cells in the absence of butanol, suggesting that the butanol adaptation of strain GK12 was due to physiological responses rather than genetic mutations. To our knowledge, no other microorganism can grow faster in the presence of such toxic and nonmetabolizable organic solvents than in the absence of those solvents.

To elucidate the adaptation mechanisms, we analyzed the fatty acid and DMA compositions of the adapted cells in comparison to the nonadapted cells. Similar to the nonadapted cells, the adapted cells increased their acyl chain lengths in a butanol dose-dependent manner (Table 3). However, considerable differences in the

compositions were observed between the nonadapted and adapted cells grown with 2.0% butanol. In particular, the proportion of short chains (myristic acid [ $C_{14:0}$ ]) in the butanol-adapted cells was much higher than in the nonadapted cells, whereas the proportion of long chains ( $C_{18:0}$  and  $C_{20:0}$ ) in the butanol-adapted cells was lower than in the nonadapted cells. It should also be noted that, when comparing the adapted cells grown with or without 2.0% butanol, there were no significant differences in the proportions of short and long chains; rather, such proportions were similar to those of the nonadapted cells grown without butanol (Table 3). These results strongly imply that 2.0% butanol-adapted cells may use alternative strategies to acquire butanol tolerance instead of altering the chain lengths of fatty acids and DMAs, at least at the concentration (2.0%) to which the cells had adapted. Interestingly, the proportions of long chains and short chains markedly increased and decreased, respectively, when the adapted cells were grown with 2.5% butanol (Table 3), showing that a 0.5% increase was critical for the collapse of the homeostatic state of the fatty acid composition.

A similar finding was reported for the adaptation of *Pseudomonas putida* to toluene (42). Changes in the membrane fatty acid compositions via the isomerization of *cis*- to *trans*-unsaturated fatty acids in response to solvent exposure were observed in nonadapted *P. putida* cells but not in adapted cells, suggesting that alternative mechanisms in the adapted cells, such as a solvent efflux system, allowed for improved solvent tolerance (43).

**Conclusions.** We successfully isolated a wide variety of butanol- and isobutanol-tolerant bacteria from environmental samples. Analyses of tolerance to these detrimental solvents using two representative strains suggested that the organisms perhaps maintain their structural integrity by increasing the extracellular capsule thickness, altering the membrane fatty acid components, and adaptation via unknown mechanisms. These findings provide further strategies for developing potential solvent-tolerant platforms for microbial fuel production.

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## REFERENCES

- Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS. 2008. Fermentative butanol production by clostridia. *Biotechnol. Bioeng.* 101:209–228.
- Schwarz WH, Gapes JR. 2006. Butanol—rediscovering a renewable fuel. *BioWorld Europe* 1:16–19.
- Jones DT, Woods DR. 1986. Acetone-butanol fermentation revisited. *Microbiol. Mol. Biol. Rev.* 50:484–524.
- Atsumi S, Hanai T, Liao JC. 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 451:86–89.
- Li J, Zhao JB, Zhao M, Yang YL, Jiang WH, Yang S. 2010. Screening and characterization of butanol-tolerant micro-organisms. *Lett. Appl. Microbiol.* 50:373–379.
- Liu S, Qureshi N. 2009. How microbes tolerate ethanol and butanol. *New Biotechnol.* 26:117–121.
- Huffer S, Clark ME, Ning JC, Blanch HW, Clark DS. 2011. The role of alcohols in growth, lipid composition, and membrane fluidity of yeast, bacteria, and archaea. *Appl. Environ. Microbiol.* 77:6400–6408.
- Ingram LO, Buttke TM. 1984. Effects of alcohols on micro-organisms. *Adv. Microb. Physiol.* 25:253–300.
- Vollherbst-Schneck K, Sands JA, Montencourt BS. 1984. Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. *Appl. Environ. Microbiol.* 47:193–194.

10. Knoshaug EP, Zhang M. 2009. Butanol tolerance in a selection of microorganisms. *Appl. Biochem. Biotechnol.* 153:13–20.
11. Qureshi N, Saha BC, Cotta MA. 2007. Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*. *Bioproc. Biosyst. Eng.* 30:419–427.
12. Woods DR. 1995. The genetic engineering of microbial solvent production. *Trends Biotechnol.* 13:259–264.
13. Kataoka N, Tajima T, Kato J, Rachadech W, Vangnai A. 2011. Development of butanol-tolerant *Bacillus subtilis* strain GRSW2-B1 as a potential bioproduction host. *AMB Express* 1:10. doi:10.1186/2191-0855-1-10.
14. Ruhl J, Schmid A, Blank LM. 2009. Selected *Pseudomonas putida* strains can grow in the presence of high butanol concentrations. *Appl. Environ. Microbiol.* 75:4653–4656.
15. Ting CNW, Wu J, Takahashi K, Endo A, Zhao H. 2012. Screened butanol-tolerant *Enterococcus faecium* capable of butanol production. *Appl. Biochem. Biotechnol.* 168:1672–1680.
16. Hanada S, Kawase Y, Hiraishi A, Takaichi S, Matsuura K, Shimada K, Nagashima KVP. 1997. *Porphyrobacter tepidarius* sp. nov., a moderately thermophilic aerobic photosynthetic bacterium isolated from a hot spring. *Int. J. Syst. Evol. Microbiol.* 47:408–413.
17. Pfennig N, Widdel F, Trüper HG. 1981. The dissimilatory sulfate-reducing bacteria, p 926–940. In Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (ed), *The prokaryotes*, vol 1. Springer, New York, NY.
18. Sekiguchi Y, Kamagata Y, Nakamura K, Ohashi A, Harada H. 2000. *Syntrophothermus lipocalidus* gen. nov., sp. nov., a novel thermophilic, syntrophic, fatty-acid-oxidizing anaerobe which utilizes isobutyrate. *Int. J. Syst. Evol. Microbiol.* 50:771–779.
19. Hungate R. 1969. A roll tube method for cultivation of strict anaerobes. *Methods Microbiol.* 3:117–132.
20. Tamaki H, Hanada S, Sekiguchi Y, Tanaka Y, Kamagata Y. 2009. Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. *Environ. Microbiol.* 11:1827–1834.
21. Ludwig W, Strunk O, Westram R, Richter L, Meier H. 2004. ARB: a software environment for sequence data. *Nucleic Acids Res.* 32:1363–1371.
22. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner, FO. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35:7188–7196.
23. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–425.
24. Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596–1599.
25. Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791.
26. Toju H, Hosokawa T, Koga R, Nikoh N, Meng XY, Kimura N, Fukatsu T. 2010. “*Candidatus Curculioniphilus buchneri*,” a novel clade of bacterial endocellular symbionts from weevils of the genus *Curculio*. *Appl. Environ. Microbiol.* 76:275–282.
27. Yamashita S, Sato M, Iwasa Y, Honda K, Sameshima Y, Omasa T, Kato J, Ohtake H. 2007. Utilization of hydrophobic bacterium *Rhodococcus opacus* B-4 as whole-cell catalyst in anhydrous organic solvents. *Appl. Microbiol. Biotechnol.* 74:761–767.
28. Reid G, Cuperus PL, Bruce AW, van der Mei HC, Tomczek L, Khoury AH, Busscher HJ. 1992. Comparison of contact angles and adhesion to hexadecane of urogenital, dairy, and poultry lactobacilli: effect of serial culture passages. *Appl. Environ. Microbiol.* 58:1549–1553.
29. Hanada S, Takaichi S, Matsuura K, Nakamura K. 2002. *Roseiflexus castenholzii* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium that lacks chlorosomes. *Int. J. Syst. Evol. Microbiol.* 52:187–193.
30. Iwabuchi N, Sunairi M, Anzai H, Nakajima M, Harayama S. 2000. Relationships between colony morphotypes and oil tolerance in *Rhodococcus rhodochrous*. *Appl. Environ. Microbiol.* 66:5073–5077.
31. Zahir Z, Seed KD, Dennis JJ. 2006. Isolation and characterization of novel organic solvent-tolerant bacteria. *Extremophiles* 10:129–138.
32. Torres S, Pandey A, Castro GR. 2011. Organic solvent adaptation of Gram positive bacteria: applications and biotechnological potentials. *Biotechnol. Adv.* 29:442–452.
33. Weber FJ, De Bont JA. 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochim. Biophys. Acta* 1286:225–245.
34. Rainey F, Andersson M, Lassila ELN, Ulrych U, Busse H, Weiss N, Mikkola R, Salkinoja-Salonen M. 2000. *Frigoribacterium faeni* gen. nov., sp. nov., a novel psychrophilic genus of the family *Microbacteriaceae*. *Int. J. Syst. Evol. Microbiol.* 50:355–363.
35. Grogan DW, Cronan JE, Jr. 1997. Cyclopropane ring formation in membrane lipids of bacteria. *Microbiol. Mol. Biol. Rev.* 61:429–441.
36. Mykytczuk NCS, Trevors JT, Leduc LG, Ferroni GD. 2007. Fluorescence polarization in studies of bacterial cytoplasmic membrane fluidity under environmental stress. *Prog. Biophys. Mol. Biol.* 95:60–82.
37. Heipieper HJ, De Bont JA. 1994. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. *Appl. Environ. Microbiol.* 60:4440–4444.
38. Heipieper HJ, Neumann G, Cornelissen S, Meinhardt F. 2007. Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. *Appl. Microbiol. Biotechnol.* 74:961–973.
39. Lepage C, Fayolle F, Hermann M, Vandecasteele JP. 1987. Changes in membrane lipid composition of *Clostridium acetobutylicum* during acetone-butanol fermentation: effects of solvents, growth temperature and pH. *Microbiology* 133:103–110.
40. Pini CV, Bernal P, Godoy P, Ramos JL, Segura A. 2009. Cyclopropane fatty acids are involved in organic solvent tolerance but not in acid stress resistance in *Pseudomonas putida* DOT-T1E. *Microb. Biotechnol.* 2:253–261.
41. Weber FJ, Ooijkaas LP, Schemen RM, Hartmans S, De Bont JA. 1993. Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Appl. Environ. Microbiol.* 59:3502–3504.
42. Neumann G, Kabelitz N, Zehnsdorf A, Miltner A, Lippold H, Meyer D, Schmid A, Heipieper HJ. 2005. Prediction of the adaptability of *Pseudomonas putida* DOT-T1E to a second phase of a solvent for economically sound two-phase biotransformations. *Appl. Environ. Microbiol.* 71:6606–6612.
43. Isken S, de Bont JAM. 1996. Active efflux of toluene in a solvent-resistant bacterium. *J. Bacteriol.* 178:6056–6058.